Eur J Vasc Endovasc Surg **29**, 470–478 (2005)
doi:10.1016/j.ejvs.2005.01.030, available online at http://www.sciencedirect.com on SCIENCE DIRECT®

Activation of P38MAPK Signaling Cascade in a VSMC Injury Model: Role of P38MAPK Inhibitors in Limiting VSMC Proliferation

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Introduction. *P38 mitogen-activated protein kinase (MAPK) has a crucial role in regulating signaling pathways implicated in the cellular events leading to restenosis. We examine p38MAPK activation in response to vascular cell injury, its biological effects and determine whether selective p38MAPK inhibitors, SB220025/SB203580, decrease vascular smooth muscle cell (VSMC) proliferation.*
Methods. *Human aortic VSMCs were cultured and wounds made on the monolayers to elicit mitogenic responses and induce p38MAPK activation. P38MAPK inhibitor pretreatment, at varying doses (1–100 μM) and treatment duration was used to block p38MAPK phosphorylation. Cytotoxicity, viability, proliferation and apoptosis were determined and expression of p38MAPK/phospho-p38MAPK was obtained by chemiluminiscent immunoblot analysis.*
Results. *Phosphorylation of p38MAPK depended on injury severity and was inhibited by both p38MAPK inhibitors, but not by SB202474, a specific antagonist of p38MAPK inhibitors. VSMCs treated with p38MAPK inhibitors showed a dose-dependent decrease in viable cell number, apoptosis and proliferation, reversing the deleterious effects of p38MAPK activation comparable to controls ($p < 0.05$).*
Conclusions. *This wound injury model activates the p38MAPK-signaling cascade in VSMC and causes cell proliferation that can be abrogated by pre-incubation with p38MAPK selective synthetic inhibitors in a time and dose-dependent manner. SB220025 used here for the first time in VSMC reveals itself to be a stronger p38MAPK inhibitor than SB203580 and being a second generation inhibitor may be the preferred drug for novel therapeutic maneuvers.*

Keywords: p38MAPK; Activation; VSMC.

Introduction

Mitogen-activated protein kinases (MAPKs) are a large family of ubiquitously expressed protein serine/threonine kinases that respond to a variety of extracellular stimuli and mediate intracellular signal transduction.[1,2] They are activated by dual phosphorylation of conserved threonine and tyrosine residues, upon receiving extracellular signals via direct upstream activators called MAPK kinases (MKKs).[3,4] Subsequently, MAPKs transmit their signals by phosphorylating downstream substrates on threonine or serine residues that are adjacent to proline residues.[3] These MAPK pathways, initiated within seconds or minutes of receiving appropriate stimulus at the cell surface, ultimately lead to the phosphorylation of nuclear transcription factors and signals transduced through them can be amplified and diversified to reach distal effectors.[3,5] MAPKs are believed to play a pivotal role in regulation of cell proliferation, apoptosis, cell differentiation, cytoskeleton remodeling and the cell cycle.

MAPKs have been implicated in VSMC proliferation, hypertrophy, and migration, processes central to the pathogenesis of vascular diseases.[4,6] They have been suggested to play a crucial role in restenosis after arterial and venous reconstructive therapy. P38MAPK is known to be strongly activated in response to vascular injury.[7] Furthermore, phosphorylation of p38MAPK dramatically stimulates its ability to phosphorylate protein substrates (Fig. 1). Since, the manifestations resulting from vessel wall injury may be mediated by the ability of p38MAPK to potentiate the cellular proliferative and reparative response of VSMCs to local stimuli, its inactivation may be a means to limit abnormal vascular cell proliferation. Therefore, selective pharmacological inhibition of

Presented at the Annual Meeting of European Vascular Surgery Society, Turkey, September 28, 2002.
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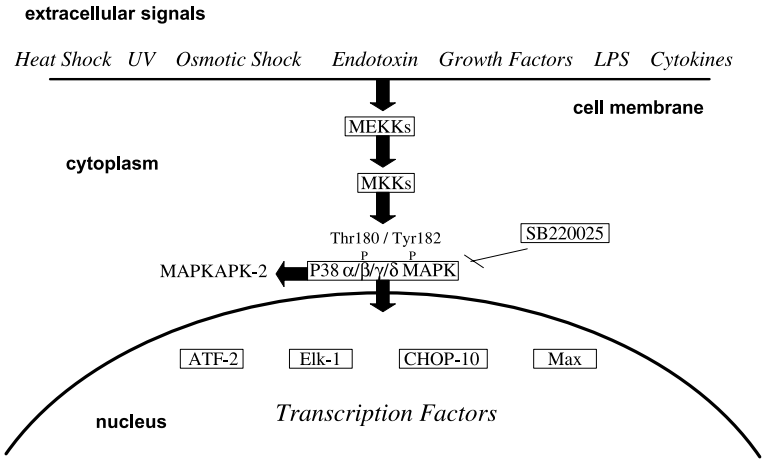

Fig. 1. Diagrammatic representation of p38MAPK signaling pathway. Abbreviations: LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinases; MEKK, mitogen-activated protein kinase/ERK kinase kinase; MKK, MAPK kinases; MAPKAPK, MAPK-activated protein kinase; ATF, activating transcription factor; CHOP, C/EBP (CCAAT/enhancer-binding protein) homologous protein.

p38MAPK may effectively decrease neointimal hyperplasia following vascular wall injury.

SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl) imidazole, is a selective inhibitor of p38MAPKs, reported to limit VSMC proliferation.[8,9] SB220025, 5-(2-amino-4-pyrimidinyl)-4-(4-fluorophenyl)-1-(4-piperidinlyl) imidazole is a new potent, more selective and specific inhibitor of human p38MAPK. While it is a second-generation inhibitor, with reportedly 2000-fold greater selectivity for p38MAPK over extracellular signal-regulated kinases (ERKs),[10] its effect has not been studied in VSMCs. We hypothesized that the significantly greater selectivity of SB220025 for p38MAPK over other kinases would be of value in developing molecular therapeutic strategies.

In this study, we investigated the dual phosphorylation of p38MAPK in response to vascular cell injury using a well-established *in vitro* wound model for p38MAPK activity stimulation in human VSMC. We examined whether SB220025 could abrogate phosphorylation of p38MAPK as compared to SB203580, and attempted to delineate molecular mechanisms in the p38MAPK-signaling cascade.

Materials and Methods

Cell culture

Human aortic vascular smooth muscle cells (VSMCs) from a single donor obtained from American Type Collection Center (Manassas, VA) were cultured in F12-K medium (ATCC), containing 2 mM L-glutamine, penicillin/streptomycin and supplemented with 10% fetal bovine serum, 0.01 mg/ml insulin, 0.01 mg/ml transferrin, 10 ng/ml sodium selenite, 0.03 mg/ml endothelial growth supplement, 0.05 mg/ml ascorbic acid, 10 mM HEPES and 10 mM TES. Cells from passages 3 to 5 were used in the study. The cells from the same passage number were used for treatment and the corresponding control. The cells were seeded on 75 cm^2 tissue culture-treated flasks (Falcon, Franklin Lakes, NJ) and grown as monolayers at 37 °C, 5% CO_2. For experiments the cells from confluent cultures were subcultured onto 96-well and 6-well tissue culture dishes (Falcon, Franklin Lakes, NJ) at a density of 3200 cells/well or 18,000 cells/well. After the cultures reached 70% confluence, the medium was replaced with serum-free medium for 24 h to attain quiescence before treatments were commenced.

Treatment

After a 24-h period, the serum-free medium was aspirated and the monolayers treated with or without medium containing various concentrations of SB220025 or SB203580. SB202474, a p38MAPK inhibitor antagonist, was used as a negative control and lipopolysaccharide (LPS) at 10 μg/ml was used as a positive control for p38MAPK activation. Stock solutions were prepared in DMSO and working dilutions in culture medium. Initially, a pilot study was done with 18 different p38MAPK inhibitor concentrations ranging from 0.1 to 100 μM, to determine the workable

concentrations of the inhibitors, the negative control and vehicle, by a cytotoxicity assay. It was determined that the relevant concentrations of SB220025 and SB203580 for further experiments on inhibition of p38MAPK, was from 1 to 30 μM. The time of exposure required for p38MAPK activation was 5 min onwards. The concentrations of 1.0–5.0 μM of inhibitors appeared to be the inflection point in all our cellular assays after which concentration there was a progressive decrease in the cellular viability and proliferation. For the *in vitro* wound injury model, VSMC were seeded in 6-well, flat-bottomed, tissue culture treated plates with 18,000 cells/well. Sub-confluent cultures were treated as described above. After 1 h of incubation at 37 °C, 5% CO_2, wounds were made with a sharp sterile blade on the VSMC monolayers to induce p38MAPK activation by vascular cell injury either with 10 or 20 cuts. Controls were with or without wounds as well as without p38MAPK inhibitor pretreatments. The following assays were performed on the cell cultures treated in this manner.

Analysis of p38MAPK phosphorylation

Protein extraction

After 0, 15, 30, 60, 120 or 360 min, monolayers were rinsed with ice-cold PBS. Laemmli sample buffer at 2× concentration (2% SDS, 20% glycerol, 0.04 mg/ml bromophenol blue, 0.12 M Tris–HCl, pH 6.8, 0.28 M β-mercaptoethanol) was added (150 ul/well). After a 5-min incubation on ice, the cells were scraped out of the culture dishes and lysed by trituration. The lysate from each well was collected into several 0.5 ml microfuge tubes on ice and immediately flash-frozen in liquid nitrogen. These were then cryopreserved at −70 °C until use.

Western blot

Equalized amounts of protein lysates (25 ug/sample) was heated to 95 °C for 5 min, cooled, loaded in each well on a 15% SDS-PAGE mini-gel (Bio-Rad, Hercules, CA) and fractionated using the Laemmli buffer system. Electrotransfer of the separated proteins was made to polyvinyldifluoride membrane (Bio-Rad) and non-specific IgG binding blocked by incubation in Tris-buffered saline containing 0.01% Tween 20 and 5% non-fat dry milk powder, for 1 h at room temperature. The membrane was sequentially probed with rabbit polyclonal antibodies, phospho-p38MAPK (Thr180/Tyr182) and p38MAPK (New England Biolabs, Beverly, MA), at 1:1000 dilution by overnight incubation at 4 °C. The phospho-p38MAPK antibody detects endogenous levels of p38MAPK only when activated by dual phosphorylation at Thr180 and Tyr182, while the p38MAPK antibody detects total levels of endogenous p38MAPK protein. Immunoreactivity was detected with Phototope–HRP Western Detection System (New England Biolabs) using enhanced chemiluminescence. Densitometric analysis of the immunoblots was performed using NIH ImageJ (Image-Pro Express v. 4.0) software. Blots were stripped for re-probing by incubating in 62.5 mM Tris–HCl, pH 6.8, 100 mM β-mercaptoethanol, 2% SDS for 30 min at 50 °C followed by two washes with phosphate-buffered saline (PBS) and 0.05% Tween, then blocking was performed. Activation of p38MAPK was calculated as the percentage dual phospho-p38MAPK to total p38MAPK immunoreactivity observed in the same sample for the same blot. Data are expressed as arbitrary units representative of three separate blotting experiments.

Cellular assays

Determination of cell viability

Cell viability was assessed using trypan blue exclusion at 48 h post-treatment. Trypsinized cells were harvested from cell culture dishes and resuspended in phosphate buffered saline. Equal volumes of cell suspension were mixed with 0.4% trypan blue dye solution. A hemacytometer was used to determine the viable cell number for each treatment group by an observer blinded to the type of treatments. The cells that excluded trypan blue represented the fraction of viable cells. For each experiment, there were triplicate wells for treatment and controls. All assays were performed three times on cells from each well. In addition, the entire experiment was independently performed for a total of three times.

Determination of cell proliferation

Cell proliferation was determined using the CellTiter96 AQ Proliferation Assay (Promega, Madison, WI), which incorporates a colorimetric method for assessing proliferation. Following serum-starvation for 24 h, monolayers in the 96-well plates were treated with media containing various concentrations of SB220025, SB203580 or SB202474. After 48 h, 20 μl (0.05:1) of the solution containing a tetrazolium compound, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and an electron coupling reagent, phenazine methosulfate (PMS), were added to 100 μl of culture medium in each well. Cultures were then incubated in humidified incubators at 37 °C, 5% CO2 for 2 h. Cell proliferation was assayed colorimetrically

at 490 nm on a microplate reader (Packard, Meriden, CT) by virtue of the red color intensity of the MTS formazan product formed by cellular degradation of MTS. Experiments were performed in quadripulate for each concentration and repeated independently three times.

Determination of apoptosis

Detection of apoptosis *in situ* was by Tdt-mediated dUTP nick end-labeling of free 3′OH DNA termini of fragmented DNA present in the apoptotic cells (TUNEL) using the ApopTag kit (Intergen, Purchase, NY) as per the manufacturer's instructions. Briefly, cells were harvested, washed with PBS and fixed with 1% paraformaldehyde/PBS for 10 min at room temperature. The fixed cell suspension was applied on slides and air-dried. The slides were equilibrated in ApopTag equilibration buffer for 5 min after which reaction buffer containing TdT enzyme and digoxigenin-dUTP was added to the slides. The slides were incubated in humidified chambers for 1 h at 37 °C in the dark. End-labeling was terminated by immersion in stop wash buffer for 20 min at 37 °C. Blocking solution containing anti-digoxigenin antibody (sheep polyclonal) conjugated to fluorescein was applied and incubated for a further 30 min at 37 °C in humidifying chambers. The antibody solution was washed away with three changes of PBS for 5 min each. End-labeling was visualized after counterstaining with propidium iodide and observing the yellow–green fluorescence under Zeiss Axiophot fluorescence microscope (Carl Zeiss Inc., Thornwood, NY). Ten random fields per slide were examined under high magnification (×1000) and apoptotic cells were counted manually. A total of 1000 cells were counted in each specimen. The incidence of apoptosis was also detected by observing the morphological markings of programmed cell death including chromatin condensation and cell shrinkage under a phase contrast microscope. Cells with these features have been confirmed to be apoptotic by electron microscopic analysis in previous studies by us.[11] Experiments were done in triplicate and three separate experiments were performed.

Statistics

The data were analyzed by *t*-test and Chi-square. Fisher's exact test was used to compare the values obtained at different concentrations to that in the controls. One-way analysis of variance (ANOVA) with post-hoc Bonferroni multiple comparison test, was performed to compare the results in the different groups. p value of <0.05 was considered statistically significant. Statistical analyses were performed using Graphpad Instat (GraphPad Software, Inc. San Diego, CA) and SPSS version 8.0 (SPSS Inc.; Chicago, IL) software.

Results

Cell viability

VSMC viability at seeding was 94–97%. After serum starvation for 24 h, the cell viability ranged from 88 to 94% in the cell cultures. At 48 h post-treatment, viability in the controls with normal media was 82–92%. VSMCs treated with p38MAPK inhibitors, SB220025 and SB203580 showed a dose-dependent decrease in total and viable cell numbers (Fig. 2). However, treatments with p38MAPK inhibitors and SB202474, did not demonstrate appreciable loss of VSMC viability except at the highest concentration i.e. 100 μM of these pharmacological agents, being 82, 83 and 77% for SB220025, SB203580 and SB202474, respectively. As depicted in the figure, there is an initial decrease in cell counts at lower doses. At 1 μM concentration of both p38MAPK inhibitors, VSMC cultures demonstrated a significant increase in cell numbers. From 5 μM onwards the cultures showed presence of cell debris, decrease in cell numbers and loss of cell viability. Maximum decrease in cell numbers was at the highest dose of 100 μM for both p38MAPK inhibitors as compared to controls ($p<0.05$). In the cultures treated with SB202474, a dose-dependent decrease in cell numbers was observed in all the concentrations of this p38MAPK inhibitor antagonist, with the lowest viability in 100 μM treated monolayers. When the effect of these pharmacological compounds on VSMC cultures are compared, 100 μM treatment of SB203580 seems to be most severe, with a decrease in cell number equivalent to 75% of untreated controls.

Cell proliferation

There was a dose-dependent effect of p38MAPK inhibitors on the serum-stimulated VSMC proliferation resulting in a decrease in viable cell numbers. Pharmacologic inhibition of p38MAPK by SB220025 and SB203580 suppressed serum-induced VSMC proliferation. Although at lower concentrations (1–5 μM), SB220025 did not affect a decrease in cell proliferation, treatments with concentrations of SB220025 above 5 μM demonstrated a significant decrease in VSMC proliferation (Fig. 3). At 1 μM

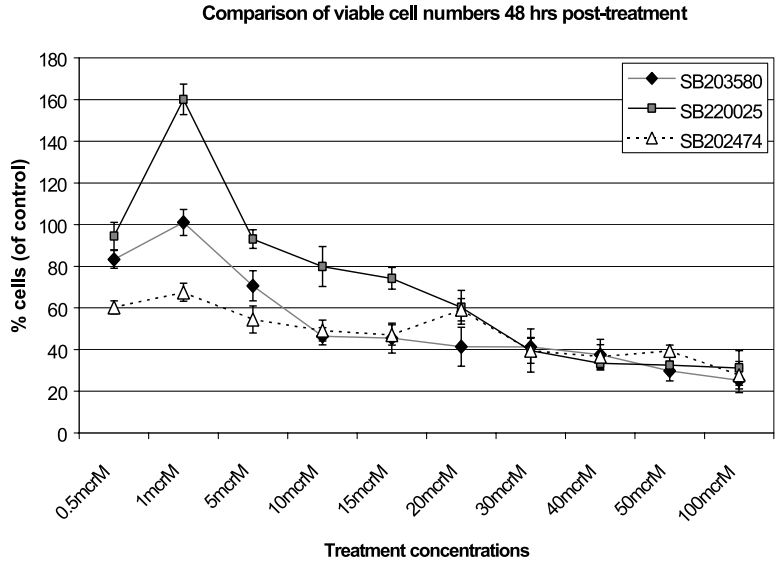

Fig. 2. Viability assays. Cell numbers were enumerated 48 h post-treatment varying concentrations of SB220025, SB203580 and SB202474 (antagonist for SB220025 and SB203580) as compared to untreated (control) as described under Section 2. Results are data from three individually treated cell culture wells of three separate experiments and are expressed as % mean ± SD of control (each value represents mean of nine data points). One-way analysis of variance gives $p < 0.01$ for 1–20 μM treatment concentrations when these pharmaceutical agents were compared.

SB220025 there was a 21% increase in proliferation as compared to the controls. While the proliferation in 0.1 and 0.5 μM SB203580 was lower than that in the untreated control, the concentration of 1 μM SB203580 demonstrated an increase in VSMC proliferation. This increase was significant and the maximum observed in any concentration used of the inhibitors and was 30% more than that in the control ($p < 0.05$). Treatment with concentrations above 1 μM SB203580 demonstrated a significant decrease in VSMC proliferation. There was no significant difference in the effect of SB202474 on VSMC proliferation.

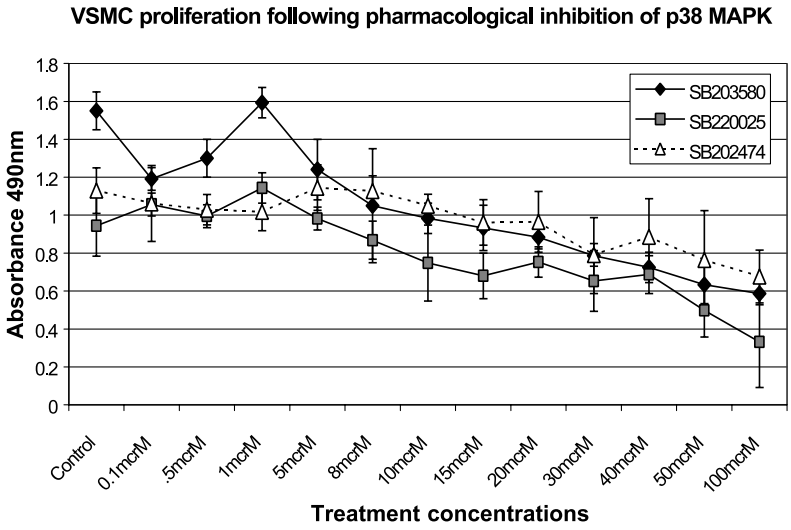

Fig. 3. Proliferation assays. VSMC seeded in 96-well plates, serum starved and treated with varying concentrations of SB220025, SB203580 and SB202474 (antagonist for SB220025 and SB203580) or left untreated (control). CellTiter96 AQ Non-radioactive Proliferation Assay was used to determine the proliferation 48 h following treatment. Results are expressed as absorbance at 490 nm. Data are results of three separate experiments with quadripulate wells/treatment/experiment and are expressed as mean ± SD absorbance at 490 nm (each value represents mean of 12 data points). One-way analysis of variance gives $p < 0.01$ for 1–20 μM treatment concentrations when these pharmaceutical agents were compared.

Analysis of p38MAPK phosphorylation

Since, the MAPKs are activated by phosphorylation of specific threonine and tyrosine residues, the phosphorylated state of any MAPK is a good measure of its activation. Assessment of the phosphorylated state in VSMCs (serum-stimulated/injury-induced activation of p38MAPK) was accomplished by Western analysis using dual phospho-specific antibody that binds to the activated p38MAPK. Percentage dual phospho-p38MAPK to total p38MAPK immunoreactivity was calculated as observed in the same sample for the same blot. Total cellular protein harvested from serum stimulated VSMCs demonstrated the presence of p38MAPK. Mechanical injury to VSMCs rapidly (within 15 min) stimulated strong phosphorylation and activation of p38MAPK (Fig. 4). LPS-stimulated p38MAPK was used as positive control. This activation of p38MAPK was time as well as dose-dependent (Fig. 4). The quantity of phospho-p38MAPK was higher in VSMC lysates from cultures injured with 20 wounds than that in cultures with 10 wounds, as indicated by dense bands on the immunoblots and densitometry. Pretreatment with p38MAPK selective synthetic inhibitors decreased the phosphorylated form of p38MAPK. When SB202474, the agonist for p38MAPK inhibitor was present in the cultures, p38MAPK and phospho-p38MAPK were again detected on the blot (Fig. 4).

Apoptosis

TUNEL assay demonstrated significant differences in the amount of cells undergoing apoptosis in the various treatment groups in both cell types. There was increase in programmed cell death in VSMC cultures as a result of serum starvation. The magnitude of apoptosis was high in cultures exposed to injury as compared to untreated controls. The maximum amount of apoptosis observed was in this group ($39 \pm 9.2\%$ cells) $p < 0.05$. Cultures treated with p38MAPK inhibitors demonstrated a decrease in apoptotic cells (Fig. 5). This decrease was not observed when SB202474 was used along with the p38MAPK inhibitors.

Discussion

A diverse array of extracellular signals, utilize MAPK signaling cascades to initiate a variety of cell signaling outcomes that are stimulus-specific.[1,2] The pleotropic potential of MAPK emphasizes the importance of tight control of their activation.[12] Since, p38MAPK is involved with promoting various processes such as cell migration, proliferation, differentiation, cell growth and cell cycle,[3,4,13–16] it was postulated that selective inhibition of p38MAPK may be an effective way of decreasing neointimal hyperplasia after vascular wall injury.[10]

The vascular response to mechanical injury involves inflammatory and fibroproliferative processes that result in the formation of neointima and vascular remodeling. Vascular injury gives rise to biomechanical stress leading to production of cytokines and proinflammatory molecules, which in turn leads to activation of p38MAPK. Some investigators are of the opinion that p38MAPK is activated in vascular walls after injury and promotes neointimal formation, and this may be due to release of several growth factors and cytokines in response of vascular wall damage.[10] In our model, VSMC injury caused multiple fold increase in the phosphorylation of p38 at the threonine and tyrosine sites. These results suggest that p38MAPK mediates the injury-induced and serum-stimulated proliferation in VSMCs. The magnitude of phosphorylation was greater for injury-induced proliferative stimulus as compared to that of serum-stimulation. The time course and pattern of activation studies suggest a time and injury-severity dependent activation. However, MAPK cascade signaling molecules are not only stimuli-specific in cell cultures, but also thought to be affected by culture cell density in some models. On the other hand, Li *et al.* did not observe any significant changes in p38MAPK activation in rat aortic VSMCs cultured at different cell densities.[17]

One of the earliest events in vascular wall injury is apoptosis, and p38MAPK has been shown to play a role in its induction.[10] Our results demonstrate that apoptosis is upregulated in the VSMC monolayers challenged with mechanical injury as compared to those without exposure to injury. The magnitude of apoptosis is higher than the apoptosis observed in serum-starved VSMC cultures. It is known that phosphorylation of MAPKs leads to the induction of apoptosis in some cell types, while in others like myocardial cells inhibition of apoptosis occurs.[18] When trophic factors were removed from neuronal and non-neuronal cell lines, apoptosis and p38MAPK activity were elevated in both cell lines.[19] Mackay *et al.* reported p38MAPK activation protects cardiac myocytes from apoptosis, which correlate with the role of p38MAPK in hypertrophic growth of cardiomyocytes, although there is an assumption that overexpression of p38MAPK along with SAPK (stress-activated protein kinase) induces cytopathic effects.[20] Hence, p38MAPK

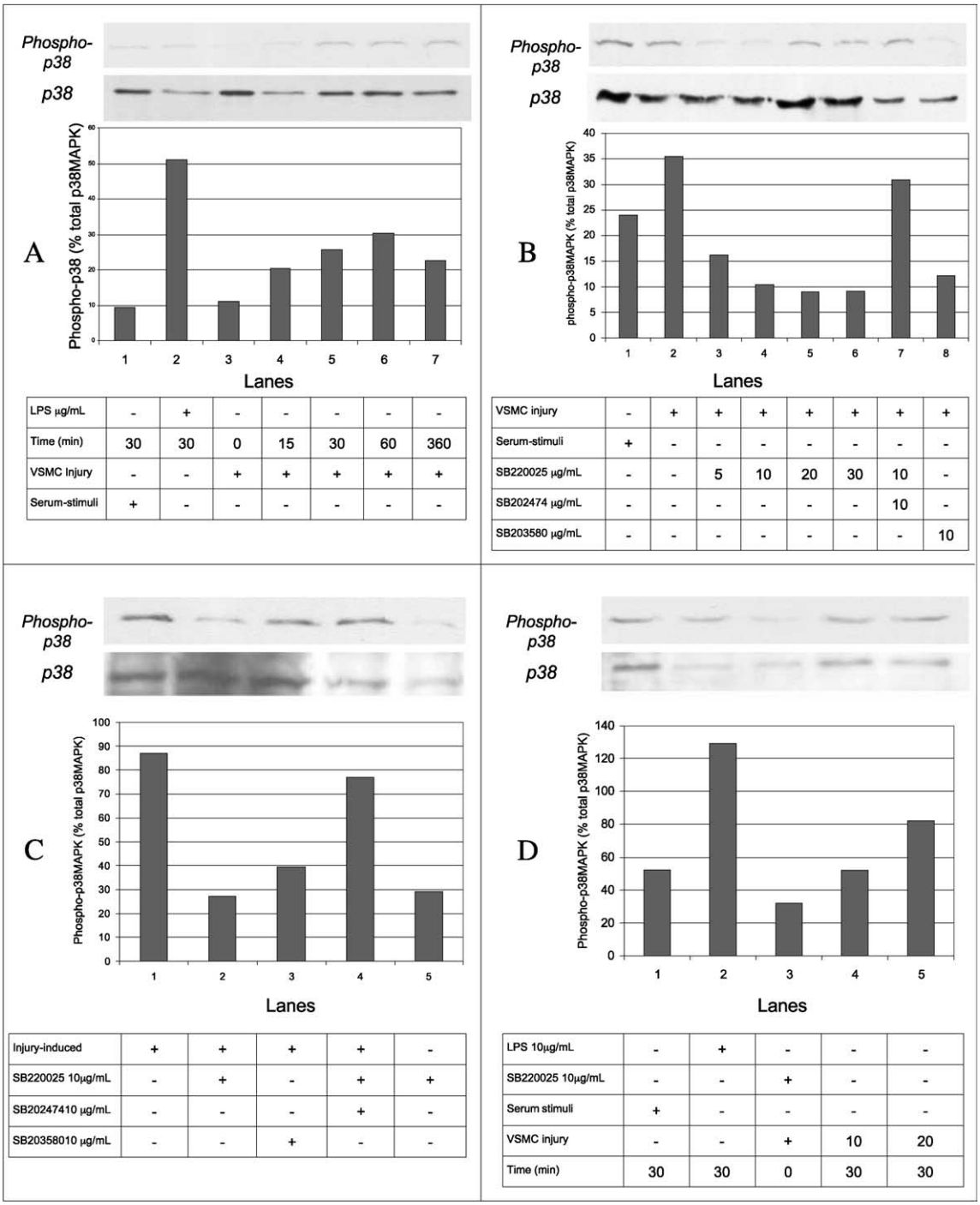

Fig. 4. Western analysis. Representative immunoblots documenting expression of p38MAPK activated by serum-stimulation, VSMC injury or LPS (lipopolysaccharide). Cell lysates of VSMCs were prepared and analyzed by chemiluminiscent immunoblot analysis as described under Section 2. Immunoblots were analyzed by densitometry. Activation of p38MAPK was calculated as the percentage dual phospho-p38MAPK to total p38MAPK immunoreactivity observed in the same sample for the same blot. Data are expressed as arbitrary units representative of three separate blotting experiments. A. Time course experiments. B. Dose response to pharmacological inhibition of p38MAPK activation at 60 min. C. Inhibition of p38MAPK activation by pharmacological agents. D. P38MAPK activation and severity of injury.

inhibitors may have the potential to reverse these cell-type specific responses to activation of p38MAPK and modulate cell-cycle regulatory pathways.

It was hypothesized that the abnormal proliferation of VSMCs might be facilitated by functional anomalies in the MAPK circuitry and pharmacological

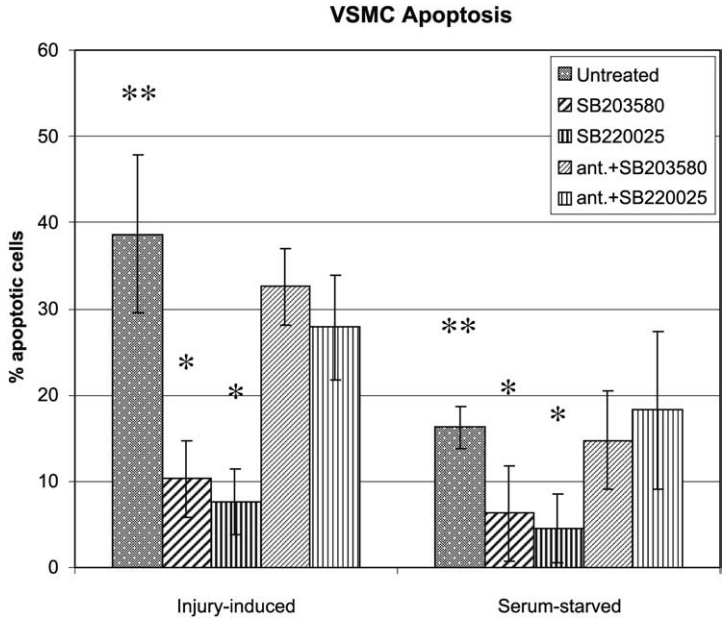

Fig. 5. Apoptosis assays. VSMCs seeded in 96-well or 6-well plates, left untreated, serum starved or monolayers injured and treated with varying concentrations of p38MAPK inhibitors/inhibitor antagonist and TUNEL assay performed 72 h post-treatment as described under Section 2. Results are expressed as % apoptosis (mean $\pm$ SD). Single asterisk (*) indicates significant difference in comparison to untreated cultures. Double asterisk (**) denotes significant p value when injury-induced and serum-starvation induced VSMC apoptosis are compared.

modulation of this pathway could be utilized for novel therapeutic ventures. Our data demonstrate that p38MAPK inhibitor SB220025 nearly ablated the proliferative growth response and suggests that it was due to p38MAPK activation. When SB203580 was used to inhibit the activation of p38MAPK in VSMCs, similar results were obtained, which concurs with observations of other investigators.[7,8] These agents blocked apoptosis stimulated by injury as well as serum-starvation in VSMCs. Immunoblot analysis revealed that the level of activated p38MAPK was significantly reduced in cell lysates of monolayers pre-incubated with SB220025 and SB203580 before injury. On the contrary, pre-incubation with a specific antagonist of these inhibitors-SB202474, did not decrease apoptosis or reduce the amount of phosphorylated p38 MAPK indicating that both SB220025 and SB203580 have the capacity to inhibit p38MAPK activation. However, SB220025 is reportedly highly selective over other kinases in comparison to SB203580 and several other p38MAPK inhibitors (500-fold over PKA, protein kinase A, 50-fold over PKC, protein kinase C and $>$1000-fold over EGFR, epidermal growth factor receptor).[21] Whereas much attention has focused upon SB203580, the utility of SB220025 in modulating p38MAPK activation has not been studied extensively. This is the first time its effect on activation of p38MAPK in VSMCs has been examined and the data suggest that it can regulate the p38MAPK-signaling cascade. In this experiment, we did not examine the concomitant expression of other MAPKs. The diversity in effects of p38MAPK on various cell types by distinct extracellular stimuli may be due to the activation of different isoforms of p38MAPK. However, here the role of these different isoforms or the effects of SB220025 and SB203580 on them were not elucidated and may be the focus of future investigation.

In conclusion, selective and specific inhibitors of p38MAPK, can downregulate the serum-stimulated and injury-induced rapid activation and phosphorylation of p38MAPK in VSMCs. Our data demonstrate that pharmacological inhibition of p38MAPK suppresses apoptosis as well as cell proliferation. A highly specific, 50–2000-fold more selective, second generation p38MAPK inhibitor, SB220025, was observed to be a stronger inhibitor and thus may be a drug of choice for therapeutic maneuvers. However, further investigation into its mechanism of action is warranted to elucidate the molecular pathways limiting VSMC proliferation.

Acknowledgements

This work was supported by a grant from Maimonides Research and Development Foundation.

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Accepted 28 January 2005
Available online 9 March 2005